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# Adaptation during propagation improves *Clostridium autoethanogenum* tolerance towards benzene, toluene and xylenes during gas fermentation

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## ABSTRACT

Benzene, toluene and xylenes (BTX) are a group of compounds detected in many crude syngas mixtures. However, BTX have been identified to negatively affect microorganisms, including acetogenic species that are capable of fermenting syngas into valuable biocommodities. In order to overcome BTX inhibitory effects, we describe stepwise adaptation in *Clostridium autoethanogenum* that leads to tolerance to up to 0.5 mM benzene, 0.21 mM toluene and 0.07 mM xylenes. This is equivalent to eightfold of that which is found in a wood gasification plant syngas stream. Fully adapted cultures matched growth, acetate and ethanol product concentrations, and CO consumption compared to the control. The results demonstrate an efficient route towards producing a highly tolerant, industrially relevant acetogenic strain.

## 1. Introduction

Microbial syngas fermentation is a novel concept for producing renewable fuels and chemicals. Syngas is a mixture primarily consisting of CO, CO<sub>2</sub>, and H<sub>2</sub> gases that are a result of thermochemical processes such as the gasification of biomass and carbonaceous materials. Syngas fermentation involves the usage of acetogenic bacteria (acetogens) that can naturally assimilate CO and/or H<sub>2</sub> as electron donors, and CO<sub>2</sub> as an electron acceptor which ultimately lead to fermentative products that include acetate, ethanol, lactate and 2,3-butanediol (Abrini et al., 1994; Tanner et al., 1993). Microbial gas fermentation is a promising alternative to the long-established, metal catalysis-based, Fischer-Tropsch process (FTP) used in the production of synthetic fuels and lubricants (Dry, 2002; Steen and Town, 2008). In contrast to the FTP, gas fermentation can use variable syngas mixtures and operates at ambient temperatures and pressures (Bengelsdorf et al., 2018).

Crude syngas invariably contains a range of impurities with fluctuating concentrations that may have inhibitory effects on gas-fermenting bacteria. Furthermore, the nature and concentration of these impurities depends on the gasification process, feedstock and gasifier type (Belgiorno et al., 2003). Remarkably, microbial processes are considered to be less sensitive to relatively low concentrations of impurities compared to the FTP's metal catalysts which are highly susceptible to poisoning (Liew et al., 2016). Nevertheless, trace amounts of impurities are still thought to negatively impact bacteria, and the effect of impurities detected in raw syngas is still not widely understood and investigated in industrially relevant acetogen species.

Commercialised microbial gas fermentation currently utilises steel mill off-gas with Lanzatech beginning demonstration plants in China with initial production volumes of 300 Mt a<sup>-1</sup> of ethanol (Stoll et al., 2020). Further initiatives are underway in Belgium and South Africa which aim to reach ethanol volumes of up to 62,000 Mt a<sup>-1</sup> (Stoll et al., 2020). All of these operations favour syngas derived from steel mills, as it is attractive for two main reasons. Firstly, a high (> 50%) CO fraction makes this a desirable feedstock for acetogenic bacteria as it can simultaneously serve as an electron donor and carbon source (Molitor et al., 2016). Secondly, steel mill syngas is considerably cleaner than syngas derived from conventional municipal waste, and biomass gasification sources, and requires fewer clean-up and conditioning steps (Molitor et al., 2016; Munasinghe and Khanal, 2011). Despite this, if syngas fermentation is to be a widely adopted option of carbon capture and bio-commodity production, a wider range of syngas sources must be considered as possible feedstocks.

Besides the main constituents of syngas, minor side components found in crude syngas fractions contain several chemical species that include, and are not limited to; ammonia, nitrous oxide(s) and dioxides, hydrogen sulphide, sulphur dioxide, cyanide, methane, ash and various traces of hydrocarbons (e.g.: ethylene, acetylene, ethane) (Xu et al., 2011). A subset of inhibitors that are prevalent in many raw syngas fractions are tars, which remain a persistent contaminant in various gasification systems. Tars cover a broad range of compounds that include polycyclic aromatic compounds (PAHs) and benzene, toluene, ethylbenzene and xylenes (BTEX, or BTX omitting ethylbenzene), of which BTX can account for up to 60–70% of the gasifier tar composition (Hernández et al., 2013).

Studies examining the effects of tars and BTX on microbial gas

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**Table 1**

Growth of *C. autoethanogenum* in the presence of varying amounts of BTX compounds, compared against the control without BTX inhibitor addition. “Similar” refers to growth similar to the control.

Cond.	Benzene (mM)	Toluene (mM)	Xylenes (mM)	Growth on fructose without BTX adaptation	Growth on CO without BTX adaptation	Growth on CO after 1st adaptation, cells from condition 7	Growth on CO after 2nd adaptation, cells from condition 10
0 <sup>a</sup>	0	0	0				
1	0.05	0	0	Similar	–	–	–
2	0.1	0	0	Similar	–	–	–
3	0.5	0	0	Similar	–	–	–
4	1	0	0	Similar	–	–	–
5	2	0	0	No growth	–	–	–
6	5	0	0	No growth	–	–	–
7	0.05	0.021	0.007	Similar	Similar	–	–
8	0.1	0.042	0.014	–	Delayed	Similar	–
9	0.2	0.085	0.028	–	Delayed	Delayed	Similar
10	0.3	0.13	0.042	–	–	Delayed	Similar
11	0.4	0.19	0.056	–	No growth	No growth	Similar
12	0.5	0.21	0.07	Similar	–	–	Similar
13	1	0.43	0.14	No growth	–	–	–
14	2	0.85	0.28	No growth	–	–	–

Dash (–) represents no experiments performed using this condition.

<sup>a</sup> Condition 0 served as control.

fermentation have observed propagation delay and suggest implementing syngas clean-up methods to prevent growth inhibition (Ahmed et al., 2006). In certain strains of *Pseudomonas* and *Bacillus*, a degree of adaptation and tolerance to BTX has been observed, which has included increasing cell-wall density, actively pumping out toxic compounds, reducing cell wall hydrophobicity and active conversion to a less toxic form (Isken and de Bont, 1998). Oswald et al. (2018) observed that the acetogen, *Clostridium ljungdahlii* could be progressively adapted to hydrogen cyanide on both sugar and syngas carbon sources. Hydrogen cyanide is a common inhibitor found in crude syngas (Oswald et al., 2018).

Short-term exposure to inhibitors during propagation is an established way for increasing robustness in *S. cerevisiae* fermentation of lignocellulosic hydrolysates (van Dijk et al., 2019). In line with this, a strategy to increase an acetogen's tolerance towards BTX could be to gradually expose acetogens to progressively higher concentrations of BTX in short-term adaptations. In this study, our aim was to demonstrate if adaptation to BTX compounds using the model acetogen, *Clostridium autoethanogenum*, can improve gas conversion rates in the presence of BTX compared to unadapted cells. Here, we examined the effects of increasing concentrations of BTX inhibitors in sugar and gas fermentations.

## 2. Materials and methods

Unless states otherwise, all chemicals were purchased from Sigma-Aldrich. CO gas used in serum flask cultivations was purchased from AGA Gas AB.

### 2.1. Microorganism and media

*C. autoethanogenum* JA1-1 DSM 10061 (ATCC 55383) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) and the cells were revived in an anaerobic workstation at 37 °C in a modified version of PETC 1754 medium (American Type Culture Collection, ATCC). The growth medium comprised per litre: 20 g 2-(N-morpholino)ethanesulfonic acid (MES), 2 g NaCl, 1 g NH<sub>4</sub>Cl, 0.1 g KCl, 0.2 g KH<sub>2</sub>PO<sub>4</sub>, 0.2 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.02 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.25 g CH<sub>3</sub>COONa, 1.5 g Casein Hydrolysate, 0.05 g Fe(SO<sub>4</sub>)<sub>2</sub>(NH<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O, 0.4 g L-Cysteine, 10 mL trace metal solution, 1 mL Wolfe's vitamin solution, and 1 mL of 0.1% w/v resazurin. The trace metal solution contained per litre: 2 g nitrilotriacetic acid, 1 g MnSO<sub>4</sub>·H<sub>2</sub>O, 0.8 g Fe(SO<sub>4</sub>)<sub>2</sub>(NH<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O, 0.2 g CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.2 g ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.02 g CuCl<sub>2</sub>·2H<sub>2</sub>O, 0.02 g NiCl<sub>2</sub>·6H<sub>2</sub>O, 0.02 g Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 0.02 g Na<sub>2</sub>SeO<sub>3</sub> and 0.02 g Na<sub>2</sub>WO<sub>4</sub>·2H<sub>2</sub>O. Wolfe's vitamin solution contained per litre: 0.002 g biotin, 0.002 g folic acid, 0.01 g pyridoxine, 0.005 g thiamine-HCl, 0.005 g riboflavin,

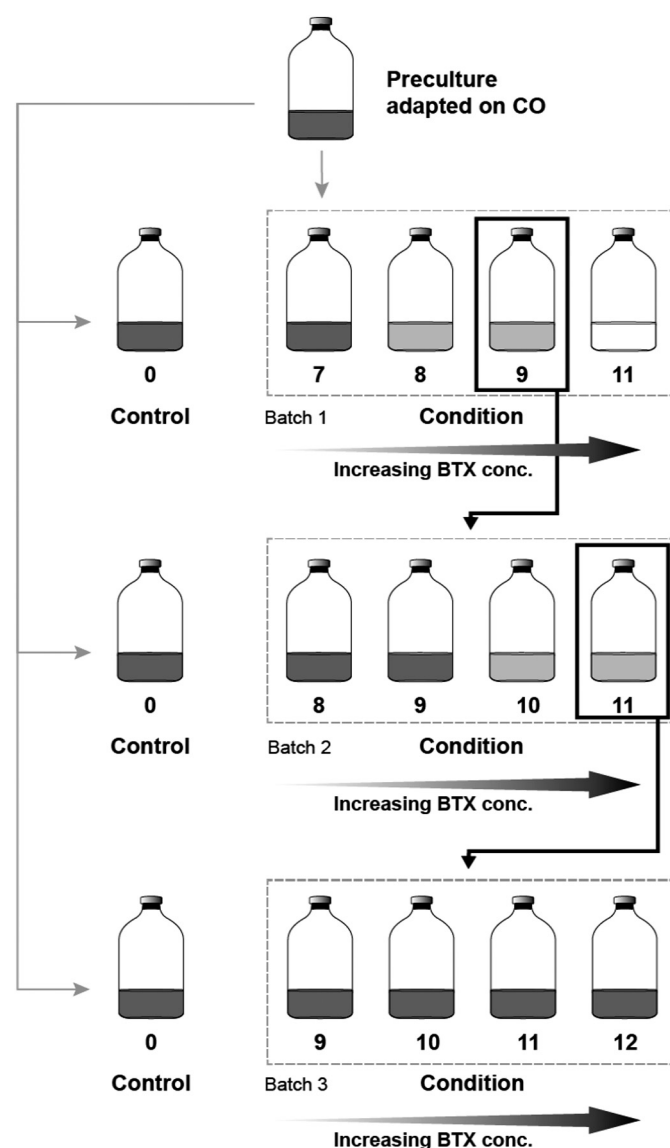
0.005 g niacin, 0.005 g Ca-pantothenate, 0.005 g cobalamin, 0.005 g 4-aminobenzoic acid, and 0.005 g lipoic acid, the final pH was buffered to 7.8. Prior to autoclaving, the medium was adjusted to pH 5.8.

Experiments were performed in 150 mL sealable serum flasks. Each flask was filled with 50 mL PETC medium and sealed with a rubber stopper. Flask headspace was replaced with pure N<sub>2</sub> by vacuum flushing using a gas-exchange system. Flasks were autoclaved and subsequently gas exchanged again with pure N<sub>2</sub> to flush out remaining traces of air.

### 2.2. Tolerance and adaptation experiments

Pre-cultures of *C. autoethanogenum* were cultured in serum flasks in PETC medium supplemented with 50 mM of D-fructose or filled with 100% CO at 2 bar absolute pressure. Flasks were incubated horizontally at 37 °C and shaken at 160 RPM. The optical density (OD) was recorded at 660 nm to avoid spectral interference from resazurin, all cultures were inoculated to a starting OD<sub>660</sub> of 0.01, and all experiments were performed in triplicate. Pre-cultures for tolerance experiment in fructose were done with *C. autoethanogenum* pre-cultured on fructose, whereas tolerance experiments using CO, were first pre-grown on CO gas to enable uniform growth and reduce lag time. The inhibitors, benzene, toluene and xylenes (mixture of ortho-, meta-, and para-isomers) were diluted using anaerobic PBS buffer and added to the experimental flasks. Experiments were conducted over a period of seven days with three 1 mL culture samples collected at the start, middle and end of the experiments.

Initial fructose-grown tolerance experiments were performed only using benzene in the following range of concentrations; 0, 0.05, 0.1, 0.5, 1, 2 and 5 mM. To assess BTX tolerance and promote adaptation, BTX compounds were introduced to serum flasks as described above. Concentrations of benzene, toluene and xylenes were based on approximate ratios of 7:3:1 that closely matched ratios found in crude syngas data derived from the Chalmers heating plant, resulting in each compound having a corresponding concentration related to this ratio, and scaling proportionally when increased (see Results and discussion, Table 1). Conditions 7 and 8 approximately resembled BTX concentrations in the Chalmers heating plant syngas fraction. At the end of each batch, individual serum flasks that were representative of tolerances at the highest BTX concentration conditions were selected and transferred into a fresh batch of serum flasks with increasing BTX concentrations (Fig. 1). The initial fructose-based experiments were performed to ensure that the effects of applied inhibitors would be seen as a direct result of these compounds, and not be potentially influenced by the organism's gas metabolism that is dependent on gas mass-transfer parameters. After successfully applying this adaptation strategy, experiments were



**Fig. 1.** BTX adaptation; *C. autoethanogenum* was first adapted on CO gas until reaching mid-exponential growth (dark grey). The preculture then inoculated CO-containing flasks, added with increasing concentrations of a BTX mixture. Cultures that represented tolerance at the highest BTX concentrations (light grey) compared to non-growth (white) were transferred into fresh serum flasks with higher BTX concentrations (light grey flask boxed in black).

repeated using CO as the sole carbon source in three successive batches.

Growth of the cells was estimated through measurement of OD<sub>660</sub> using a GENESYS 20 Visible Spectrophotometer (Thermo Scientific, UK), and supernatant samples were collected for HPLC analysis using a Dionex Ulti-Mate 3000 HPLC system (Thermo Fischer Scientific). Volumes of 20 µL were injected into a Bio-Rad Aminex HPX-87H (Biorad) column, which was kept at 80 °C and had a uniform flow rate of 5 mM H<sub>2</sub>SO<sub>4</sub> mobile phase at 0.8 mL/min. Detection was done through a refractive index (RI) detector. Headspace pressure measurements were determined using a DPG 110 hand-held pressure gauge (Omega Engineering) to assess basic rates of CO consumption.

### 3. Results and discussion

#### 3.1. Tolerance to BTX compounds

*C. autoethanogenum* was chosen as a biocatalyst for its well-characterised performance in steel mill-based syngas fermentation processes and is

considered a model acetogen (Liew et al., 2016). Our initial aim was to determine whether *C. autoethanogenum* could adapt to and tolerate levels of BTX inhibitors represented in a “real-world” industrial syngas stream. This project selected syngas from the Chalmers heating plant, which is a 2–4 MW (10–20 dry tonnes of biomass/day) wood waste gasifying plant, and analysis of its syngas impurities revealed a BTX fraction totalling 10.04 mg/L (Thunman et al., 2018). These compounds were detected in a raw syngas stream at 350 °C using a solid-phase adsorption method (Israelsson et al., 2013). The abundance of each compound in the BTX mixture of this syngas stream was analysed to be as follows: benzene 6.63 mg/L (0.085 mM), toluene 2.79 mg/L (0.03 mM) and xylenes 0.62 mg/L (0.006 mM) (combined ortho-, meta-, and para-isomers), rounding up these amounts, results in the BTX component ratio being roughly 7:3:1 (Berdujo Vilches et al., 2016). The BTX fraction was the most abundant amongst other chemical impurities analysed, with benzene being the main constituent. Therefore, to determine the inhibitory effects of benzene only, *C. autoethanogenum* was grown in ideal conditions using fructose as a carbon source.

When grown on fructose in the presence of benzene, (cond. 0–6, Table 1), *C. autoethanogenum* showed no differences in growth amongst concentrations up to 1 mM benzene. Similarly, ethanol and acetate production titres were not negatively affected by benzene addition (Fig. S1, supplementary material). However, 2 mM and 5 mM of benzene resulted in no appreciable growth or product formation.

In the next step, all of the constituent BTX species were combined to assess if a more pronounced inhibitory effect would be observed. The combination of several inhibitors was reasoned to lead to synergistic effects. Indeed, when exposing the cells to BTX, a greater growth inhibition was seen. For concentrations above 1 mM benzene; 0.43 mM toluene and 0.14 mM xylenes (cond. 13), no growth was observed on fructose, alongside no production of acetate or ethanol (Table 1, conditions 0, 7, 12–14).

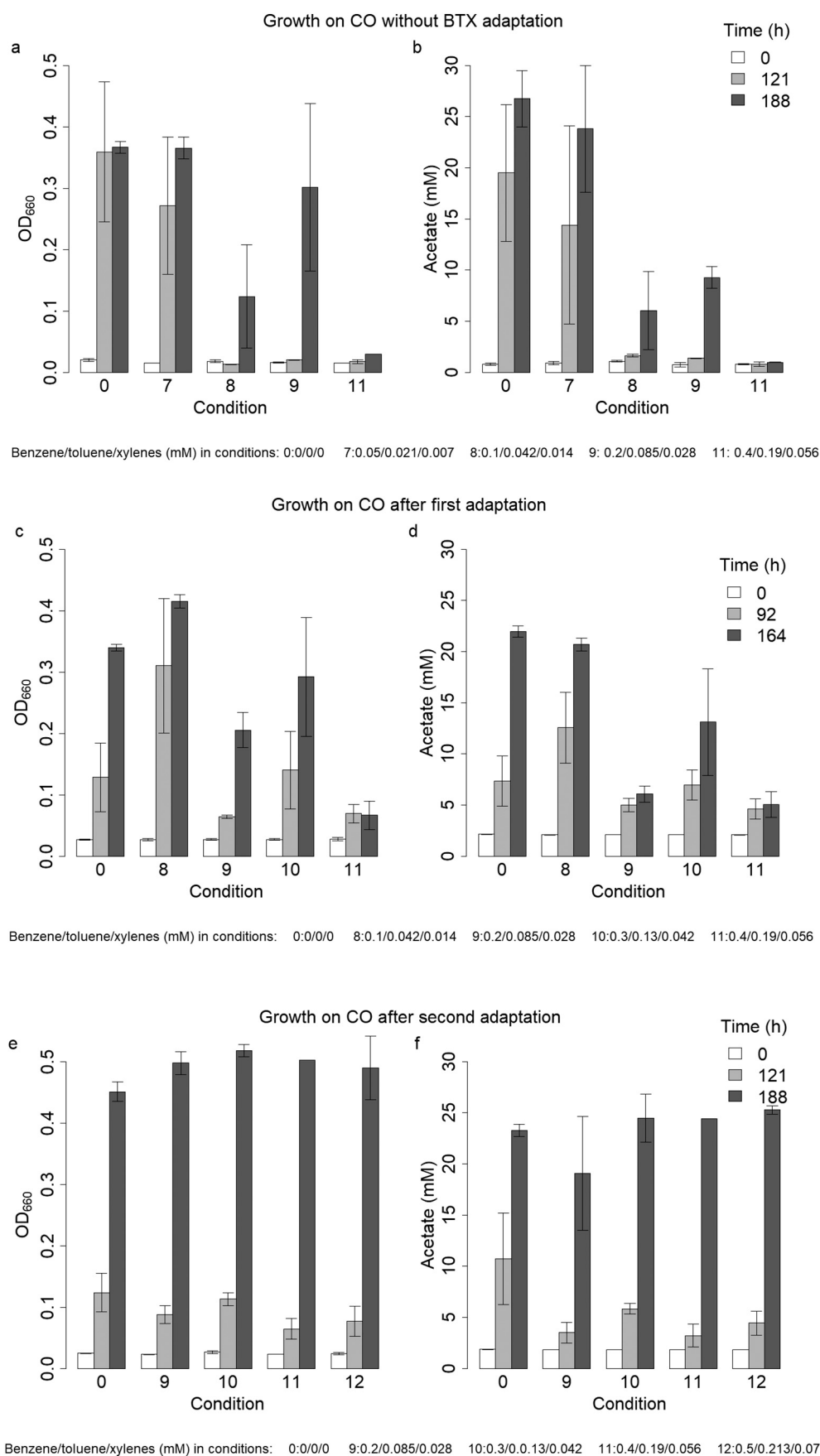
Thereafter, the carbon source was changed from fructose to CO, in order to determine BTX tolerance of *C. autoethanogenum* during autotrophic growth. After *C. autoethanogenum* was adapted on CO, cells were transferred into serum flasks with inhibitors added and incubated for 7 days. Up to 0.2: 0.085: 0.028 mM (cond. 9) of BTX was tolerated with delayed growth, but already 0.1/0.042/0.014 mM (cond. 8) led to a notable delay in growth compared to the absence of inhibitors (Table 1, Fig. 2a–b). No growth was observed when cultures were supplemented with 0.4/0.19/0.056 mM (cond. 11) of BTX compounds. Accordingly, Ahmed et al. (2006) reported cell dormancy induced when the cells were exposed to syngas, most likely caused by tars.

It should be noted that *C. autoethanogenum*'s auto- and heterotrophic growth employs different metabolic pathways which may influence the level of tolerance towards environmental stressors, but share similar energetics as reported in *C. autoethanogenum* (Marcellin et al., 2016). It is generally regarded that acetogenic growth is faster under heterotrophic conditions than autotrophic, which we noted when comparing growth of *C. autoethanogenum* on fructose vs. CO. In our study, tolerance towards BTX inhibitors was initially much lower using CO as a sole carbon source in comparison to growth on fructose, emphasising the need for a stepwise propagation scheme allowing for improved tolerance.

#### 3.2. Adaptation strategy towards improving BTX tolerance

Low inhibitor concentrations were initially used to gradually adapt the cells to grow on CO in the presence of BTX compounds. Cells grown in the presence of 0.05/0.021/0.007 mM (cond. 7) BTX compounds were transferred into a fresh serum flask containing inhibitors at increased concentrations as described in Fig. 1. The adaptation towards BTX inhibitors showed a clear improvement in tolerance, with delayed growth observed in 0.3/1.3/0.42 mM (cond. 10) of BTX, exceeding the previous tolerance level (Fig. 2c–d). Gas consumption was improved, approaching control conditions. Acetate production was also improved (Fig. 2d), but not yet matching control conditions without inhibitor addition.

To determine if adaptation could be further improved, cells from the culture with 0.3/1.3/0.42 mM BTX (Cond. 10) were transferred into new



**Fig. 2.** Batch 1. a,c,e) Biomass formation (through optical density measurement) and b,d,f) acetate production of cultures grown on PETC 1754 medium with CO and various concentrations of benzene, toluene and xylenes (see Table 1 for list of conditions). *C. autoethanogenum* was adapted for CO growth prior to tolerance experiments. Error bars represent SEM of 3 replicates.



serum flasks containing increased BTX concentrations. Interestingly, the tolerance towards BTX was now markedly improved (Fig. 2e–f). Growth was observed in all applied concentrations of inhibitors including the highest; 0.5/0.21/0.07 mM, (Cond. 12). Moreover, headspace pressure dropped from 2 to 0 bar at the end of fermentation, indicating that applied CO was fully consumed in all conditions suggesting that *C. autoethanogenum* was unaffected by the inhibitors.

Assessing hydrocarbon toxicity towards bacteria can use the logarithm of a solvent's partition coefficient ( $P$ ) in a standard octanon to water mixture ( $\log P$ ), which is used to assess compound hydrophobicity (Inoue and Horikoshi, 1991).  $\log P$  values of BTX are 2.13, 2.68 and 3.16 respectively. In general terms, the higher the  $\log P$  value ( $> 4$ ), the more lipophilic a compound is towards a microorganism's membrane, but is less water soluble, so overall less toxic. Conversely, compounds with  $\log P$  values between 1 and 4, are more water soluble and accumulate relatively more in the membrane leading to cell death (Isken and de Bont, 1998). Regarding the present study, all three compounds have relatively low  $\log P$  values, and in theory can accumulate detrimentally in the cell membranes, but our results suggest that *C. autoethanogenum* manages to tolerate concentration up to 8-fold than what is typically detected in a wood-based gasifier.

In our study, step-wise adaptation was used to allow cells to grow in the presence of high amounts of BTX. Adaptation is the native ability of microorganisms to evolve to selection pressures in their environments. This is exploited and refined in strain development using adaptive laboratory evolution (ALE) which involves growing a microorganism under a specific and increasing selection pressure over many cell generations, that eventually leads to beneficial mutations improving growth and fermentation performance. ALE has been successfully exemplified in the acetogen, *Sporomusa ovata*, which can gradually oxidise toxic methanol with CO<sub>2</sub> over a two month period, increasing growth rates and CO<sub>2</sub> conversion into acetate (Tremblay et al., 2015). Our approach demonstrated that simple and repetitive sub-culturing techniques over a relatively short period (approx. 3 weeks, or 18 cell generations) can improve tolerance to common inhibitor compounds. In the final adaptation experiments, growth delay and inhibition was entirely absent alongside comparable fermentative product titres and substrate consumption to the control. In contrast to previous observations (Ahmed et al., 2006), where the presence of tars led to lower acetate production and higher ethanol production in *C. carboxidivorans*, we noted an increased acetate production (Fig. 2b,d,e) and a decreased ethanol production as cells were adapted to higher BTX concentrations (Fig. S2, supplementary material). The acetate/ethanol redistribution upon adaptation should be studied further alongside the synergistic effects of other syngas inhibitors.

It can be assumed that it is possible to further increase tolerance towards BTX, but in practical terms, acetogens need only to tolerate inhibitor conditions found in gasifiers. In real-world conditions, many gasification plants must adhere to strict environmental regulations and as a consequence, emissions must be processed to meet standards of chemical production processes with well-established methods already possessing efficient means of BTX removal (Arena, 2012). In terms of fermentation, this is an advantageous outcome as cleaner syngas feedstocks will contribute towards better microbial productivity.

#### 4. Conclusions

In this study, a propagation strategy was developed to allow *C. autoethanogenum* to ferment CO, with BTX concentrations exceeding over eightfold of what is commonly detected in the Chalmers gasification plant's syngas output. A similar stepwise propagation on syngas can be expected to allow for efficient fermentation of untreated industrial syngas using other model acetogens. We expect that this approach will be very useful for developing efficient fermentation of various syngas feedstocks.

#### Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biteb.2020.100564>.

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